

EFFECTS OF LONG TERM SUSTAINED ELEVATION OF CGMP LEVELS ON CELL DEATH AND FIBROSIS IN THE HEART OF A RAT MODEL OF TYPE 2 DIABETES

Cardiovascular complications are the major cause of death in patients with type 2 diabetes. Cardiomyopathy develops during progression of diabetes, characterized by progressive damage of the heart muscle tissue and specifically, the loss of functional heart cells, the cardiomyocytes that are gradually replaced by fibrotic tissue, collagen fibers and other extracellular matrix components, profibrotic factors, and myofibroblasts, the key cells in fibrosis.

Among the potential cardioprotective factors, nitric oxide and its downstream product, cGMP, are emerging as known endogenous antifibrotic agents in the urogenital and vascular systems, however, their cardiac effect may be related more to the vasodilator action than the antifibrotic mechanism.

An animal study is underway to determine whether the pharmacological long-term sustained elevation of cGMP levels may reverse fibrosis in the heart of type 2 diabetic rats. Three groups of male rats of 5 months of age ($n=8/\text{group}$) were selected from a larger study: 1) control non-diabetic ZDF lean rats, untreated; 2) diabetic ZDF fa/fa rats, untreated; 3) diabetic ZDF fa/fa rats treated orally with sildenafil in the drinking water for two months, to elicit a sustained increase in cGMP. Upon completion, the rats were sacrificed, and the heart's left ventricle was divided into four sections; two were embedded in paraffin and two were frozen.

Estimations of the fibrotic area by histochemistry and quantitative image analysis (QIA) with Picrus Sirius red for collagen, and α -smooth muscle actin by immunohistochemistry and QIA for myofibroblast content, revealed a significant 2 and 2.5 fold-increase respectively in the diabetic as compared to the lean animals, that was significantly reduced by sildenafil. TUNEL/QIA assays for programmed cell death and troponin T for cardiomyocyte content are in progress. These preliminary results suggest that long-term continuous use of PDE5 inhibitors may act as antifibrotic and cardioprotective in the diabetic heart, but must be confirmed and complemented with other tests.

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INTRODUCTION

About 16 million Americans have diabetes mellitus, a metabolic disorder that results in hyperglycemia. Approximately 90% to 95% of diabetes sufferers have type 2 diabetes. Diabetes is characterized by the ineffective use of insulin by the body and hyperglycemia. Prolonged high elevations of blood sugar levels lead to blood vessel damage. The endothelial cells, which line blood vessels, intake more glucose, resulting in the formation of glycoproteins, which cause the thickening and expansion of the basement membrane, the structure that supports endothelial cells.

Approximately 30% of type 2 diabetes patients experience heart failure and about two thirds of them will die from cardiovascular diseases. Diabetic cardiomyopathy is the progressive damage and/or death of the myocardium (heart muscle tissue) and is accompanied by the loss of the functional heart cells (cardiomyocytes). Fibrosis, the formation of excess fibrous connective tissue, develops as a means to repair the damage. Recent studies suggest that nitric oxide and its downstream product, cyclic guanosine monophosphate (cGMP), are endogenous and pharmacological antifibrotic agents in the urogenital and vascular systems, which are potential cardioprotective factors prior to and following re-establishment of the blood flow to the heart (ischemia/reperfusion) after myocardial infarction. Vasodilator action is assumed to have

immediate cardioprotective effects, but in long-term, it may be due to antifibrotic mechanisms.

The goal of our research conducted through the NIDDK STEP-UP program was to determine in a rat model of type 2 diabetes mellitus whether the pharmacological long-term sustained elevation of cGMP levels might reverse fibrosis in diabetic cardiomyopathy.

METHODS AND MATERIALS

The experiment consisted of three groups of male rats of 5 months of age ($n=8/\text{group}$): control non diabetic ZDF lean rats, untreated; diabetic ZDF fa/fa rats, untreated; and diabetic ZDF fa/fa rats administered orally in the drinking water with sildenafil for two months, in order to generate a sustained increase in cGMP. Upon completion, the rats were sacrificed, and the left ventricle of the heart was divided into four sections from the base to the apex; two sections were embedded in paraffin and two were frozen.

The tissue sections were deparaffinized and used for immunohistochemistry through three methods: Picro-Sirius red, TUNEL assay, and α -smooth muscle actin (ASMA).

Picro Sirius Red staining was performed on each group to determine collagen deposition. The nuclei of the cells were stained with hematoxylin and the collagen was stained with Picro-Sirius red. The resulting background

(cytoplasm) appeared yellow and in some instances pale pink.

TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) was done to determine the apoptotic index. The TUNEL method detects DNA fragmentation that results from apoptosis (programmed cell death), using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to strand breaks of cleaved DNA. The cleavage sites are then detected using DAB, which displays a brown color and then counter stained with hematoxylin, which stains the cell nuclei.

ASMA determined myofibroblast content. Tissue samples were stained with a substrate and then counterstained with hematoxylin.

Following staining, slide sections were photographed using a microscope at 20 \times magnification. Ten pictures were taken per field per slide. The images were subsequently analyzed via quantitative image analysis (QIA) using Image Pro Plus software. Positive areas were counted as well as negative to determine the percentage of positive results.

RESULTS

ZDF fa/fa rats developed considerable hyperglycemia showing type 2 diabetes that was not affected by continuous long-term treatment with sildenafil. Untreated control ZDF lean rats were slightly hyperglycemic upon arrival (6.5 months of age) but became hyperglycemic at sacrifice (2 weeks later), possibly caused by stress.

The results indicated that the experiment involving α -smooth muscle actin (ASMA) showed a significant differentiation between the diabetic and silde-

nafil group ($P < .001$). There was little difference between the lean and sildenafil group ($P > .05$), while the lean vs diabetic group showed a significant discrepancy ($P < .01$). Overall, myofibroblast content in the heart, estimated by immunocytochemistry for ASMA (α -smooth muscle actin) and quantitative image analysis (QIA) was very low in the lean rats, increased by over 2.5-fold in the untreated diabetic rats and was nearly normalized by sildenafil.

Picro-Sirius red experimentation showed a significant difference between the lean and diabetic groups ($P < .001$), while the lean and sildenafil groups only indicated a slight difference ($P < .01$). The sildenafil and diabetic group did not show a significant difference ($P > .05$). The level of cardiac collagen was minimal in the lean rats, but was 2-folds higher in the diabetic fa/fa rats, and was reduced by 20% by sildenafil.

Cell death, indicated by apoptotic bodies with the TUNEL assay, was virtually negligible in all groups, and was difficult to discriminate from red cells.

DISCUSSION

The ZDF fa/fa rats had moderate cardiac fibrosis as shown by collagen and myofibroblast accumulation, which is in agreement with kidney fibrosis previously determined by this laboratory in the same set of untreated diabetic and lean rats, and higher than the one detected in the penile corporal smooth muscle tissue of these animals.

Long-term treatment with sildenafil ameliorated this cardiac fibrosis, presumably by the elevation of cGMP levels, as had been hypothesized from the effects previously observed by this

laboratory in the penile corpora cavernosa smooth muscle

These results are preliminary and need confirmation by other procedures on available specimens.

RESOURCES

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